A NEW PARAMETER FOR THE QUANTITATIVE MEASUREMENT OF THE TOXICITY OF CIGARETTE SMOKE*

by

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Parameter of the toxicity of cigarette smoke - Van Dongen

Mr. Chairman, Members of the Society:

The measurement of the toxicity of cigarette smokes is most commonly done with the all-or-non response of death, and the so-called LD_{50} is one of the more popular expressions among them.

The use of an all-or-non response in predicting the toxicity of treatments has at least two drawbacks. First, it is inherently less efficient than a graded response; and second, the experiments are terminal to the animals and thus expensive.

The above-mentioned reasons suggest the need for indirect toxicological methods, whereby a pharmacological response rather than lethality is used. Such a pharmacological response, however, should be related to toxicity and its measurement should be quantitative and simple.

The recently published salivation method in mice, developed by us, meets the above-stated requirements (fall meeting ASPET, 1969). Not only is the method non-traumatic to the animals, quantitative and simple, but it also accommodates the measurement of both direct and after-effects to treatment, and it permits the measurement of salivary performance on large numbers of mice simultaneously. This last feature makes it a suitable screening tool. Finally, it was established that the exposure of mice to cigarette whole smokes inhibits salivary secretion and that the extent of salivary inhibition is positively related to acute toxicities on the basis on experimental evidence with brands and smoke concentrations.

Although the exposure to cigarette smoke generally results in a salivary inhibition, the effect was, however, by no means consistent and reproducible. And since reproducibility is essential for any good methodology, it is the aim of this paper to examine the effect of a variety of factors on the salivary performance of mice; the results of which might aid in establishing improved experimental conditions to obtain consistent salivary inhibitions, predictive for acute toxicity of cigarette smoke.

The specific purpose of this study, therefore, is to examine the following factors of salivary inhibition: (a) whole smokes and gasphases, (b) high salivating and low salivating individuals of the same strain of mice, (c) a wide and narrow dilution range, and (d) the time after exposure.

The method to measure salivation in mice will be shown now.

Slide I shows a mouse in a stanchion by its permanently-worn collar. The mouse bits on a wire bit. A narrow cotton strip, pretreated

with pH indicator, is attached to the bit and turns from yellow to purple upon exposure to saliva. The wetted distance of the colored band is measured in mm and represents the amount of saliva excreted.

The next slide shows that a large number of mice can be studied simultaneously. Significant differences in salivary performance exist among various strains of mice. High-salivating C57BL mice. Low-salivating AKR mice.

To perform the salivation experiments, a large herd of male C57BL mice was available. The animals were approximately 24 weeks old, had been housed for some time six to a cage with their collars on. They had been adapted previously to the salivation procedure and were all in good health.

The salivary performance of mice was determined five times with a one-hour interval between measurements. The first and second measurements served to establish the control salivary performance of individual mice during that particular day. A ten-minute smoke exposure was applied immediately prior to the third measurement which will thus indicate the immediate effect to exposure. The fourth and fifth measurements were performed to examine any after-effects to treatments.

Smoke exposures were applied with the Walton-reverse smoking machine. The conditions in the smoking machine were standardized to 35 mm puffs of two seconds duration taken at intervals of 58 seconds. The smoke would persist for 15 seconds in the inhalation chamber and then be expulsed in 3 seconds followed by a fresh air purge for 40 seconds. Ten puffs were studied in each case, using the first five puffs of each of two sets of cigarettes, smoked without interruption in the one puff per minute cycle.

In the gasphase experiment, a cambridge filter was inserted between the proximal end of the cigarettes and the inhalation chamber.

The following ten treatments were applied: no machine exposure, machine exposure with air, or any one of four smoke dilutions from two cigarette brands A or B. The smoke dilutions were 1 to 5.4, 1 to 8.0, 1 to 16 and 1 to 32.0.

The cigarette brands A and B were selected on the basis of their tar content, nicotine content, and acute toxicities. Brand A has a high tar and high nicotine content, along with high acute toxicity. Brand B has a low tar, low nicotine content, along with low acute toxicity. The cigarettes were equalized in length to 65 mm and climatized at 60% relative humidity in a descicator for at least several days until immediately prior to their actual use in the smoking machine.

The next slide shows the experimental layout of a two-day experiment with whole smokes on 72 mice. On the first day, the experiment started with determing a first and second salivation measurement on 36 mice. These mice were then rankordered according to salivary performance and systematically assigned to any one of six treatment groups. The various treatments, applied immediately prior to the third salivation are thus done on very equal groups of mice. The second day's experiment on different mice was done the same, except for different smoke dilutions. Such a two-day experiment was repeated four times. Thus this whole smoke study includes approximately 72 x 4 x 5 = 1440 salivation measurements.

The next slide presents the layout of the entire salivation experiment with whole smokes. It is noteworthy that any smoke exposures can be compared validly with two types of controls, namely, first with "no exposure" and "machine exposure only," but second also with their own salivary performances during the first and second measurements since it had been shown that non-significant differences in the salivary performance of mice exist during the day.

The experimental design just described for whole smoke will be employed identically to study the effect of gasphases on salivary inhibition.

The statistical analysis of data was done primarily using the analysis of variance technique.

The next slide shows the salivary performance of mice upon exposure to various dilutions of whole smokes from brand A. The abcis represents the time for exposure, in hours, and the ordinate the level of salivary performance, expressed in mm of boundary displacement. Note the approximate constancy over time and the non-significant differences between the two controls. These curves represent the salivary performances with 1 to 32, 1 to 16, 1 to 8 and 1 to 5.4 dilutions of whole smokes from brand A. Obviously a very dramatic salivary inhibition which is significantly more pronounced in size as well as in duration with higher smoke concentrations.

The next slide shows the salivary performance of mice upon exposure to whole smokes from brand B. It shows a similar pattern as was seen with brand A, although its dose dependence is less distinct.

The next slide compares the salivary inhibitory effects of brands A vs. b, at each of the four whole smoke dilutions. The highest smoke dilution (1 to 32) is represented in the upper left figure. The lowest (1 to 5.4) in the lower right figure.

Comparison of the four figures shows clearly the increased salivary inhibition with increased smoke concentrations for each brand. Any systematic, differential inhibition between brands A and B is dilution dependent. There is no difference between the brands at the 1 to 32 dilution. An inconsistently higher salivary inhibition of brand A vs brand B was found at the 1 to 16 dilution, but consistently higher salivary inhibitions by brand A vs B were obtained with th 1 to 8.0 and the 1 to 5.4 dilutions.

Note further that the inhibitory after-effects to whole smoke exposures can extend over a period of several hours, which is quite different from what we will see later on with gasphase exposures.

The next slide summarizes the statistical reliability of the just shown results. From the list of factors that could have an effect on salivary performance, the following were found to be highly significant: smoke concentration, time after exposure, and the "concentration x brand" and "concentration x measurement" interactions.

We will now present the results from the gasphase experiments.

The next slide shows the effect on salivary performance of four dilutions of gasphases from brand A. The salivary inhibition is less pronounced in degree and duration and also not so obviously dose-related, relative to what was seen with whole smoke previously.

The next slide shows the salivation-inhibitory effects to gasphases from brand B. Again, less dramatic than its whole smoke counterpart.

The next slide compares the four gasphases of both brands at four concentrations. It shows that the salivary inhibitions are less pronounced than those with whole smokes, that they are not clearly dose-dependent, and that no systematic differences in salivary inhibition can be observed between the two brands.

Thus, from the standpoint of predicting the toxicity of tobacco smokes, the use of gasphases should be regarded as inadequate, and we will exclude gasphase data, therefor, from further consideration.

We will now focus our attention on the aspects which deal with the salivation level of individual mice, minimal smoke concentration, and optimal time after exposure for salivation measurement. These questions will be examined and statistically tested on the existing set of data.

The experimental mice within each treatment group were separated into those with "high" and those with "low" salivary performance on the basis of their second measurement.

The next slide shows the salivary performance of "high" vs "low" salivating individuals upon exposure to any one of four concentrations of whole smoke from brand A. Note that the salivary performance of low salivators is affected only to a minor degree. On the other hand, the high salivators show dramatic salivary inhibitions, the degree and duration of which are related to the smoke concentration applied.

The next slide shows the salivary performance data for high vs low salivators upon exposure to whole smokes from brand B. Note the approximate pattern of effects as was observed with brand A.

Thus the experimental evidence with two brands indicates that high salivators are more suitable than low salivators to predict toxicity on the basis of salivary inhibition.

The next slide shows the degree of success in differentiating between whole smoke from brand A and brand B on the basis of salivary inhibition of high salivators. There is no differential salivary inhibition between the brands at the 1 to 32 dilution. The 1 to 16 dilution appears to be the threshold whereby whole smoke from brand A becomes more inhibitory than that from brand B. There is a clearcut differential salivation-inhibitory effect between brands A and B with the two highest smoke concentrations.

With respect to time after exposure, to measure salivary inhibition for predictive purposes of toxicity, the slide suggests that one-hour after exposure is most optimal.

A statistical analysis was performed to examine the validity of the various differences shown graphically. The size of the F-values obtained for "brand" and "dilution" will determine the best combination of experimental conditions.

The next slide shows a table with calculated F-values under each of the 12 experimental conditions. A glance at the figures indicates the presence of larger F-values with high than with low salivators. Note further that, except for one, all are higher when the highest dilution is excluded. On the basis of dilution alone, 0, 1 or 2 hours after exposure would all be significant. However, the additional requirement to differentiate among brands narrows the optimal hour after exposure down to

one at 1 hour. Note further that the dilution effects are, under our experimental conditions, much more important to salivary inhibition than the effect of brand. This indicates, among others, that for valid brand comparisons, the smoke concentrations have to be standardized rigidly.

In Summary:

C57BL mice were exposed to four dilutions of whole smokes or gasphases from two brands of cigarettes and their salivary performance was determined immediately after, and one and two hours after exposure. Brand A has high tar and high nicotine content along with a high acute toxicity, and brand B has a low tar and low nicotine content along with low acute toxicity.

The predominant effect of the exposures to whole smokes and gasphases was that of a salivary inhibition. The salivary inhibition is more reproducible and more pronounced with whole smokes than with gasphases. To predict toxicity of cigarette whole smokes, high-salivating mice should be exposed to smoke concentrations of 1 to 16 or higher, and salivary performance should be measured one hour after exposure.

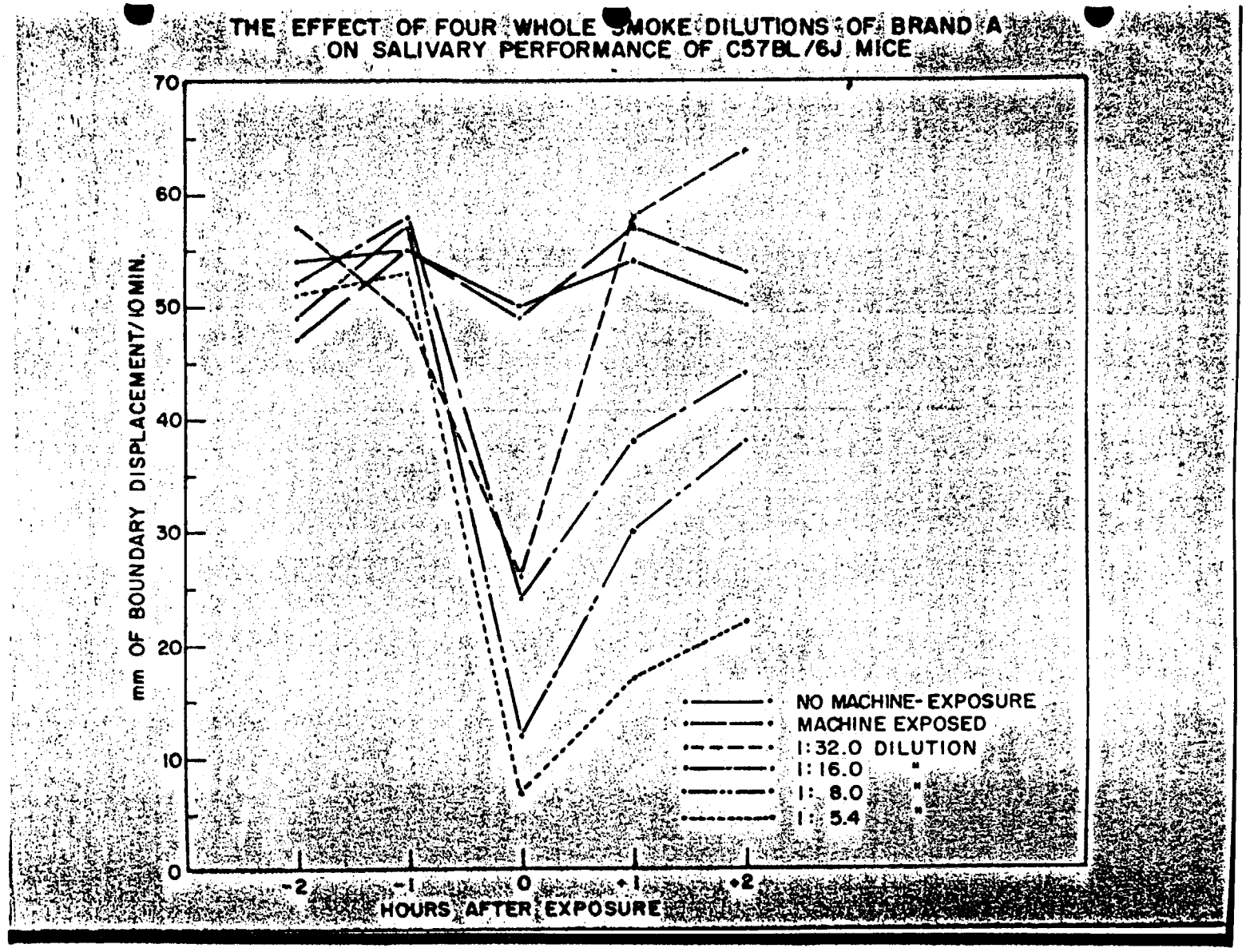
The establishment of these experimental conditions suggest that the degree of salivary inhibition can now be used to predict acute toxicity of cigarette smokes

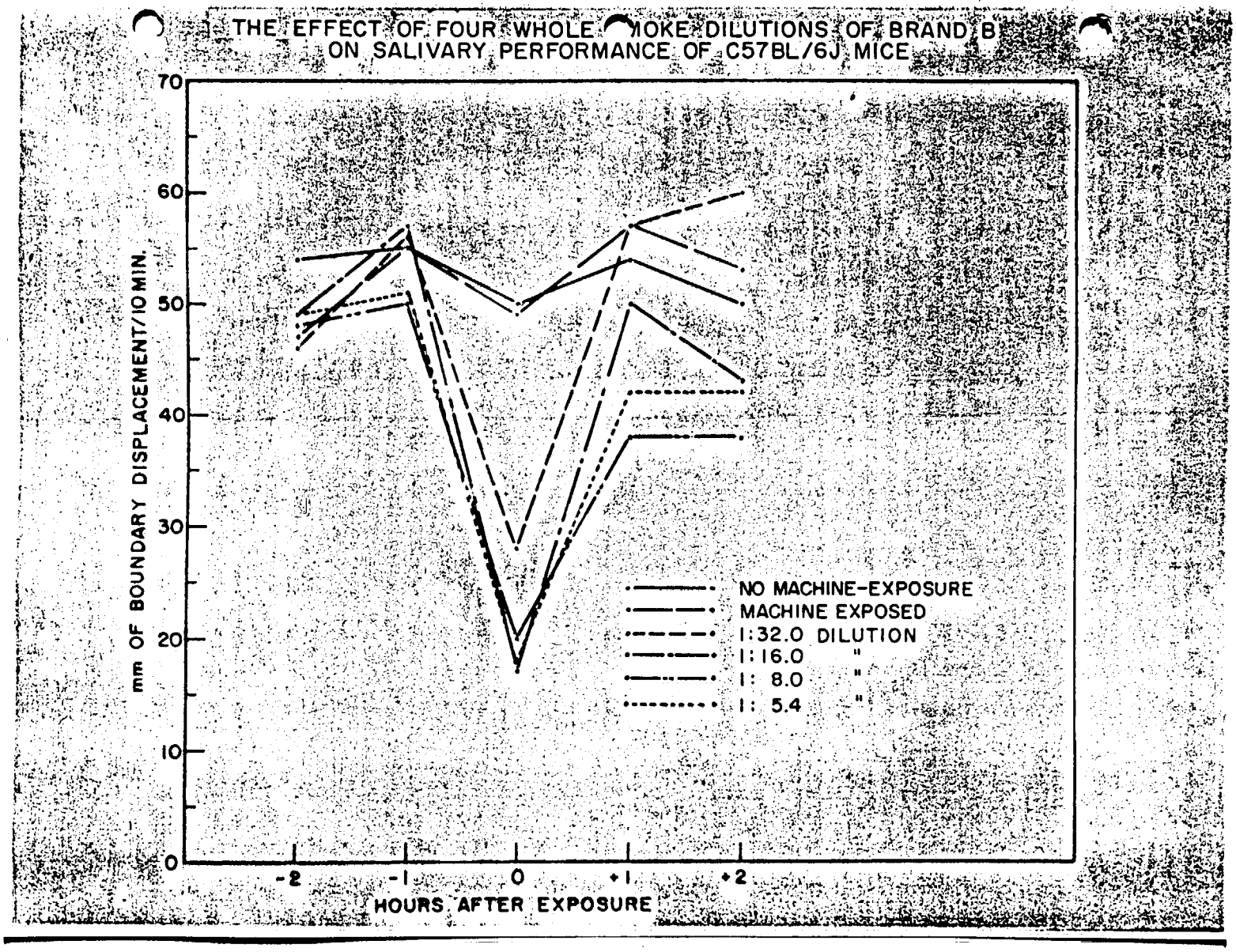
EXPERIMENTAL DESIGN OF TWO-DAY EXPERIMENT TO STUDY THE EFFECTS OF FOUR DILUTIONS OF WHOLE SMOKES FROM TWO CIGARETTE BRANDS ON THE SALIVARY PERFORMANCE OF MALE C57BL/6J MICE

Day	Treatment	Dil.	No. of Mice	Measurement				
				1	2	3	4	5
I	No machine exp.	--	6					
	Machine exp.	--	6					
	Brand A	1:32.0	6					
	" "	1:16.0	6					
	Brand B	1:32.0	6					
	" "	1:16.0	6					
II	No machine exp.	--	6					
	Machine exp.	--	6					
	Brand A	1:8.0	6					
	" "	1:5.4	6					
	Brand B	1:8.0	6					
	" "	1:5.4	6					

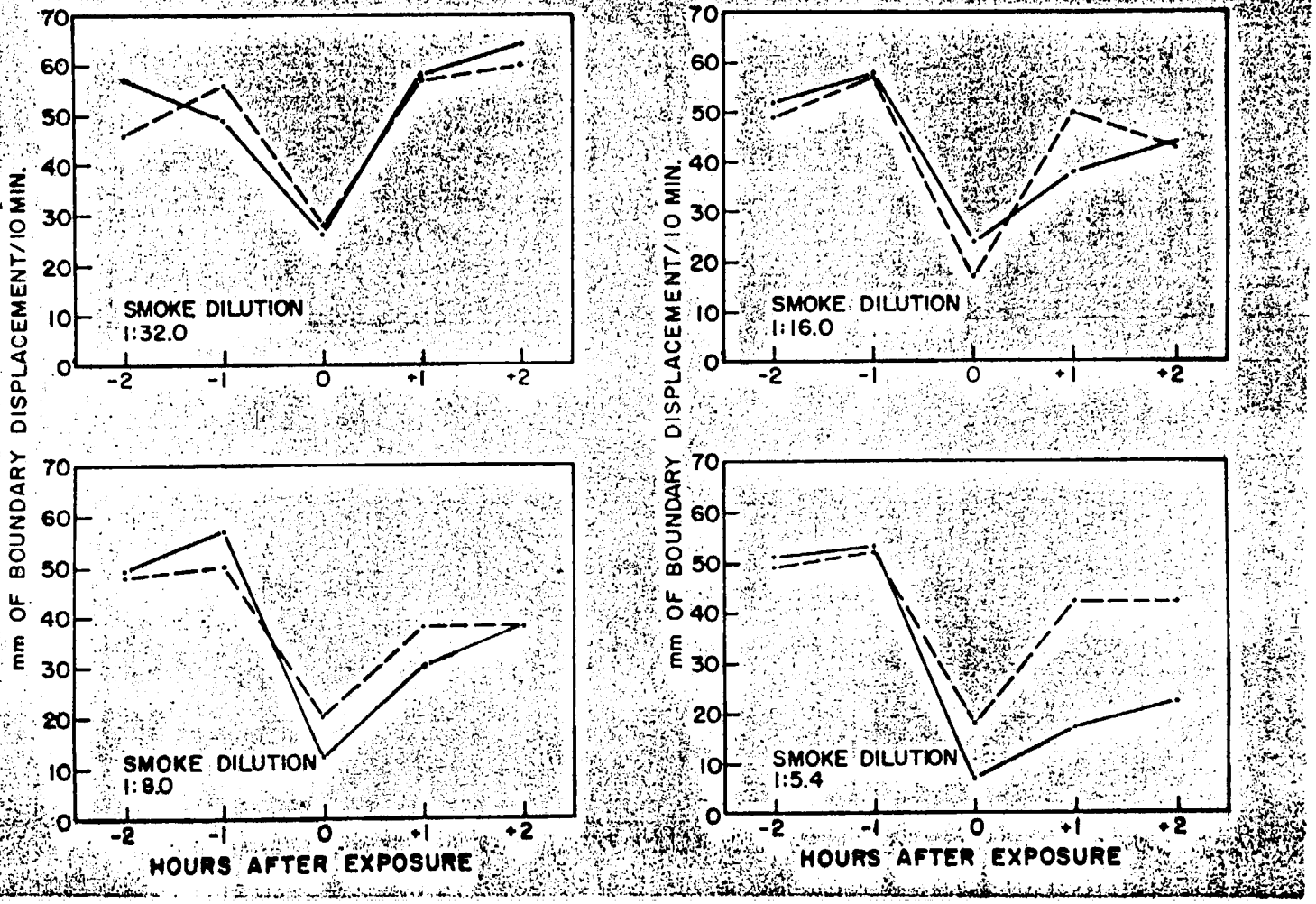
EXPERIMENTAL DESIGN TO STUDY THE EFFECTS OF FOUR DILUTIONS OF WHOLE SMOKES FROM TWO CIGARETTE BRANDS ON THE SALIVARY PERFORMANCE OF MALE C57BL/6J MICE

Treatment	Dil.	No. of Mice	Measurement				
			1	2	3	4	5
No machine exp.	--	48					
Machine exp.	--	48					
Brand A	1:32.0	24					
" "	1:16.0	24					
" "	1: 8.0	24					
" "	1: 5.4	24					
Brand B	1:32.0	24					
" "	1:16.0	24					
" "	1: 8.0	24					
" "	1: 5.4	24					





COMPARISON OF THE SALIVARY PERFORMANCE OF C57BL/6J MICE, UPON EXPOSURE TO FOUR DILUTIONS OF WHOLE SMOKE FROM BRANDS A ——• vs B– – –•

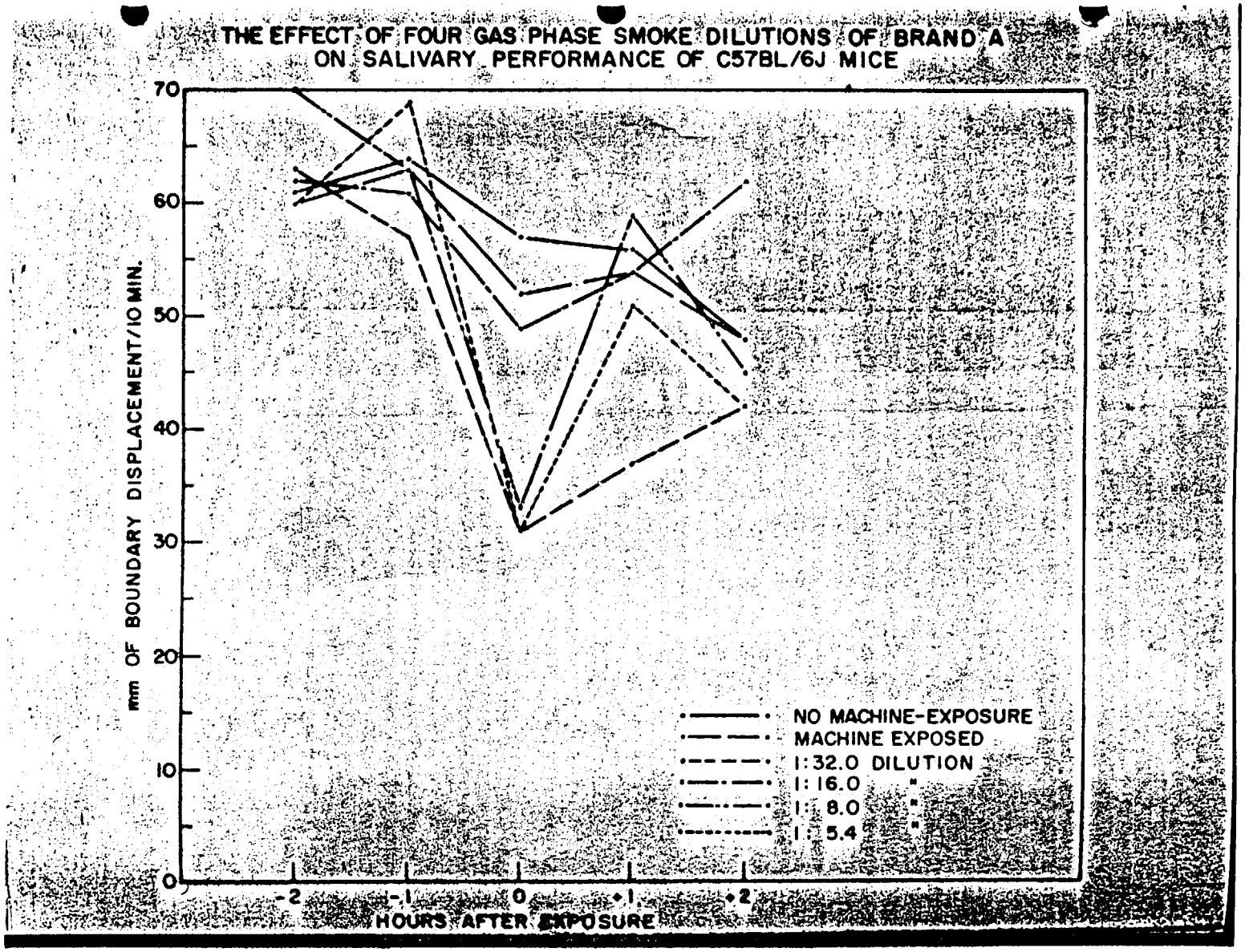


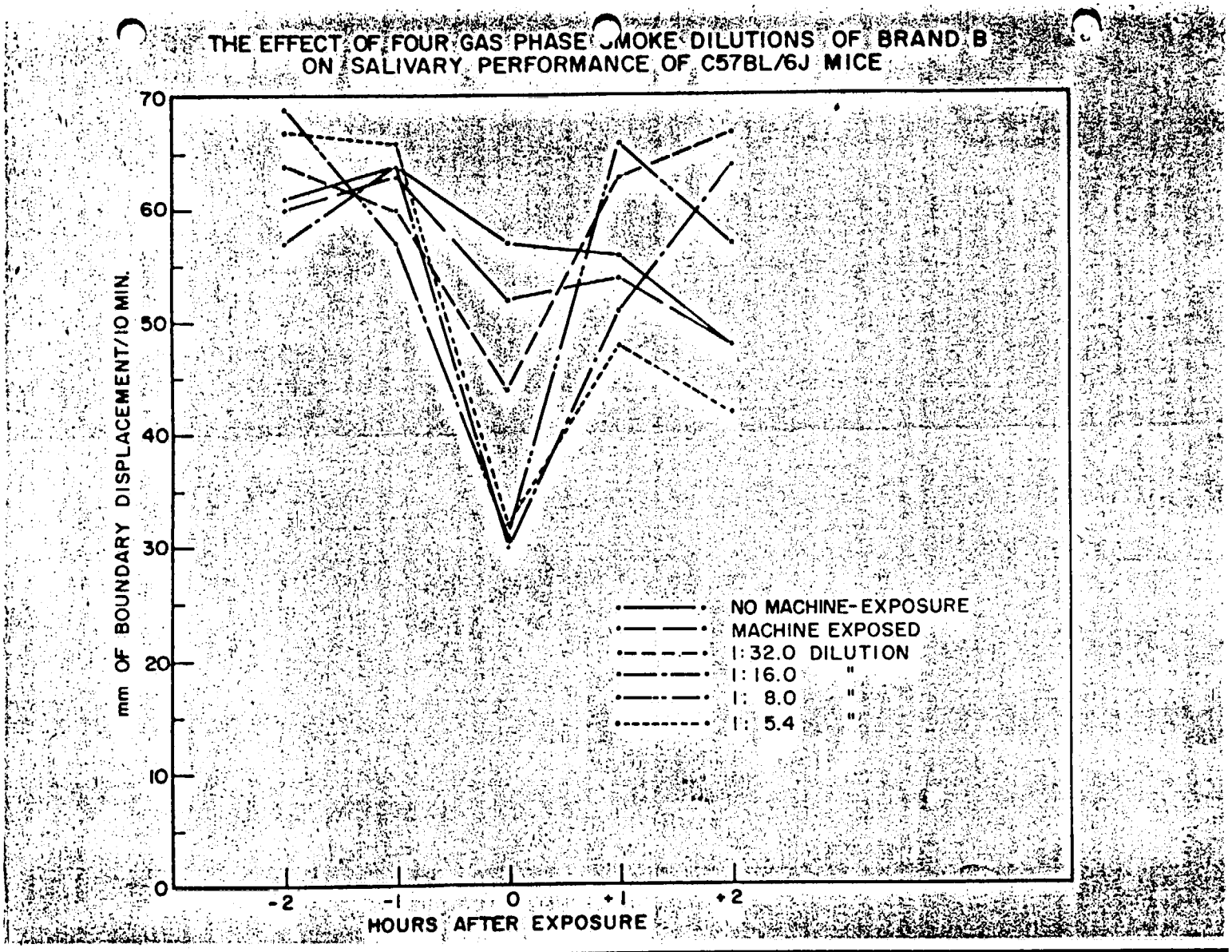
ANALYSIS OF VARIANCE

The Effects of Various Dilutions of Whole Smokes from Two Brands of Cigarettes on the Salivary Performance of Male C57BL/6J Mice (0, 1, and 2 hours after exposure)

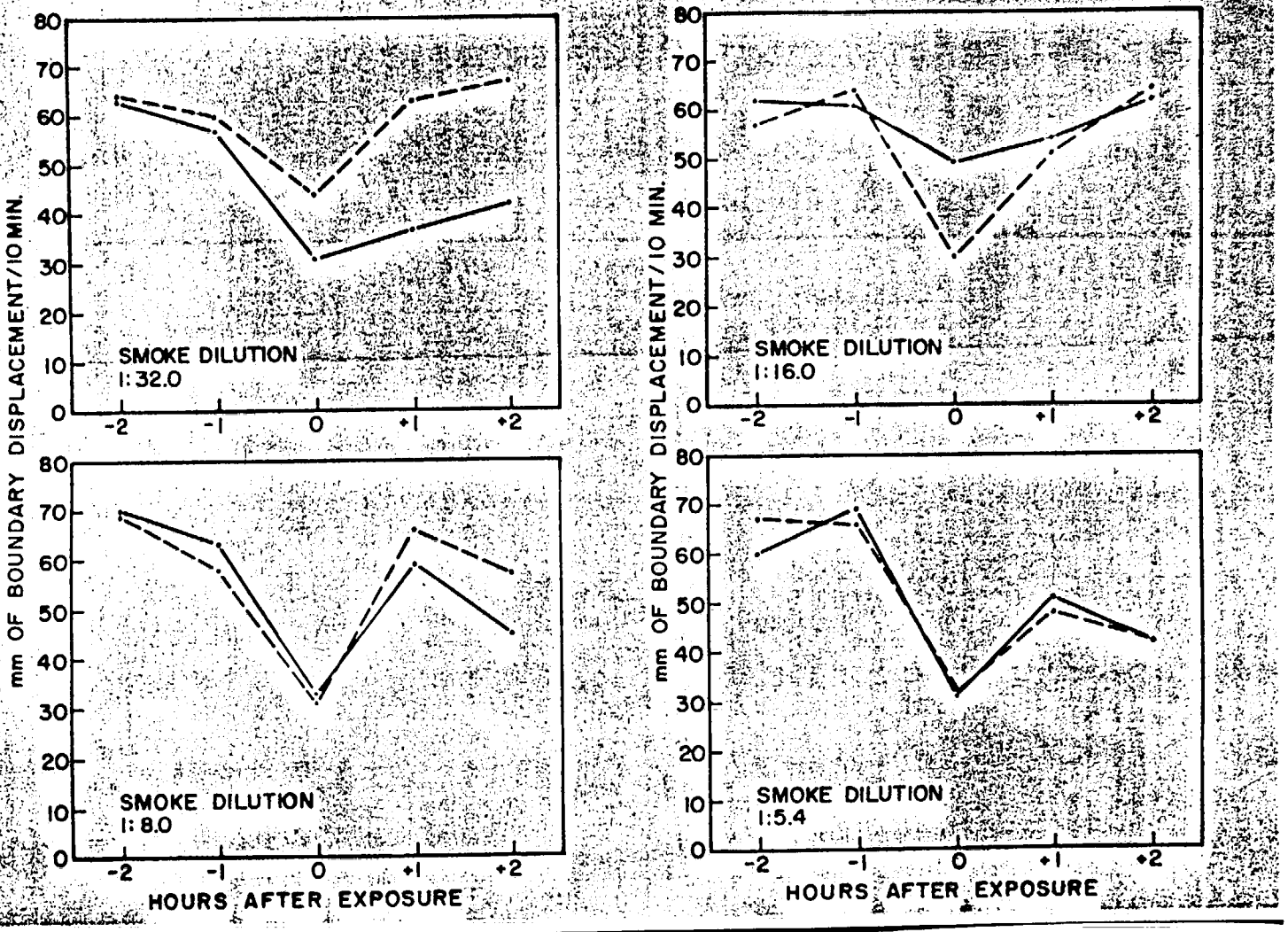
Source	DF	MS	F_{cal}	$F_{95\%}$	$F_{99\%}$
Total	815				
Brand(B)	1	1,095	1.53	3.84	6.64
Concentration(C)	5	20,159	28.08**	2.21	3.02
Measurement(M)	2	26,201	36.49**	3.00	4.61
B x C	5	2,589	3.61**	2.21	3.02
B x M	2	344	0.48	3.00	4.60
C x M	10	2,143	2.98**	1.94	2.51
B x C x M	10	553	0.77	1.94	2.51
Error	780	718			

** significant at the 1% level of probability

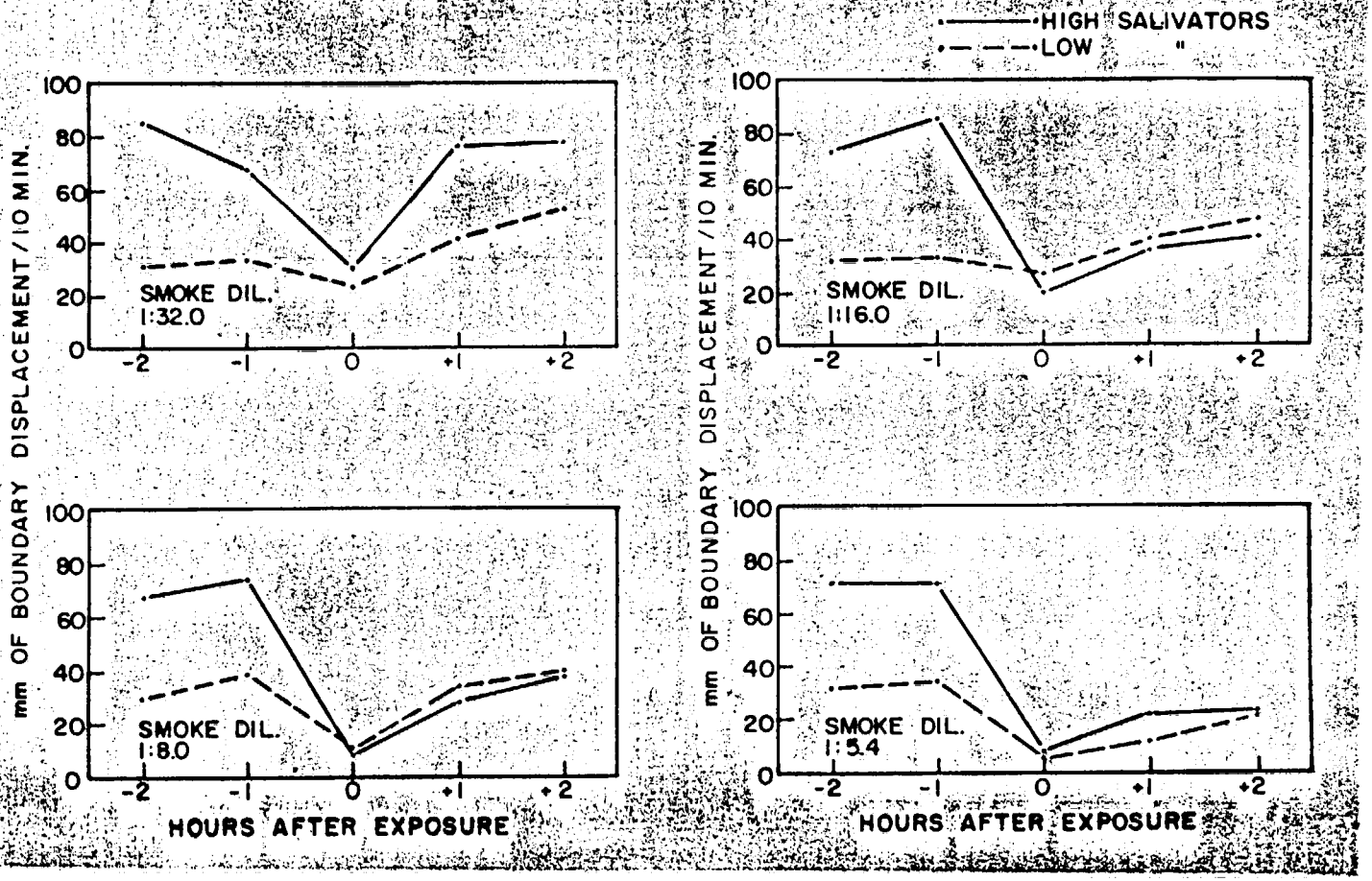




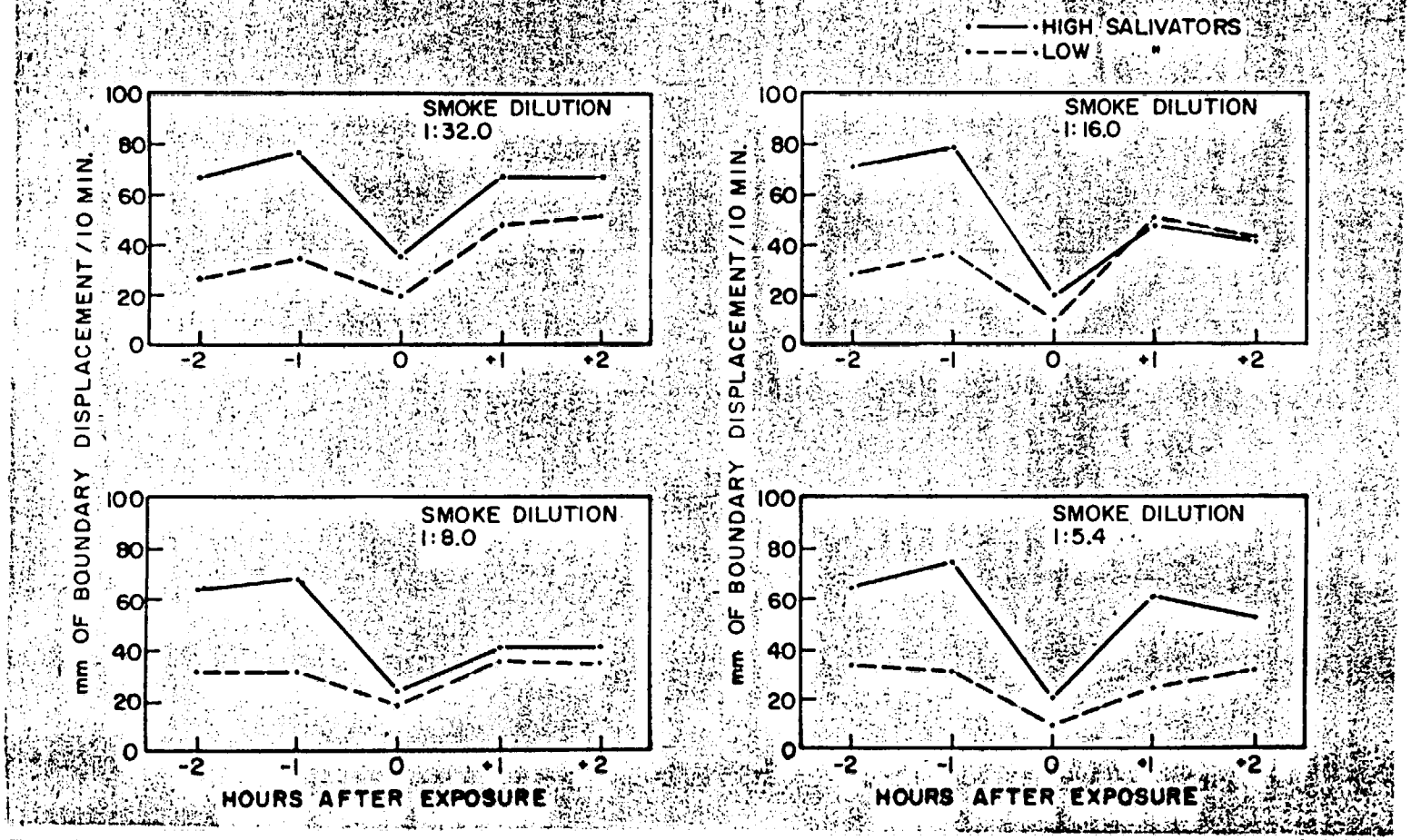
COMPARISON OF THE SALIVARY PERFORMANCE OF C57BL/6J MICE, UPON EXPOSURE TO FOUR DILUTIONS OF GAS PHASE SMOKE FROM BRANDS A •——• vs B •——•



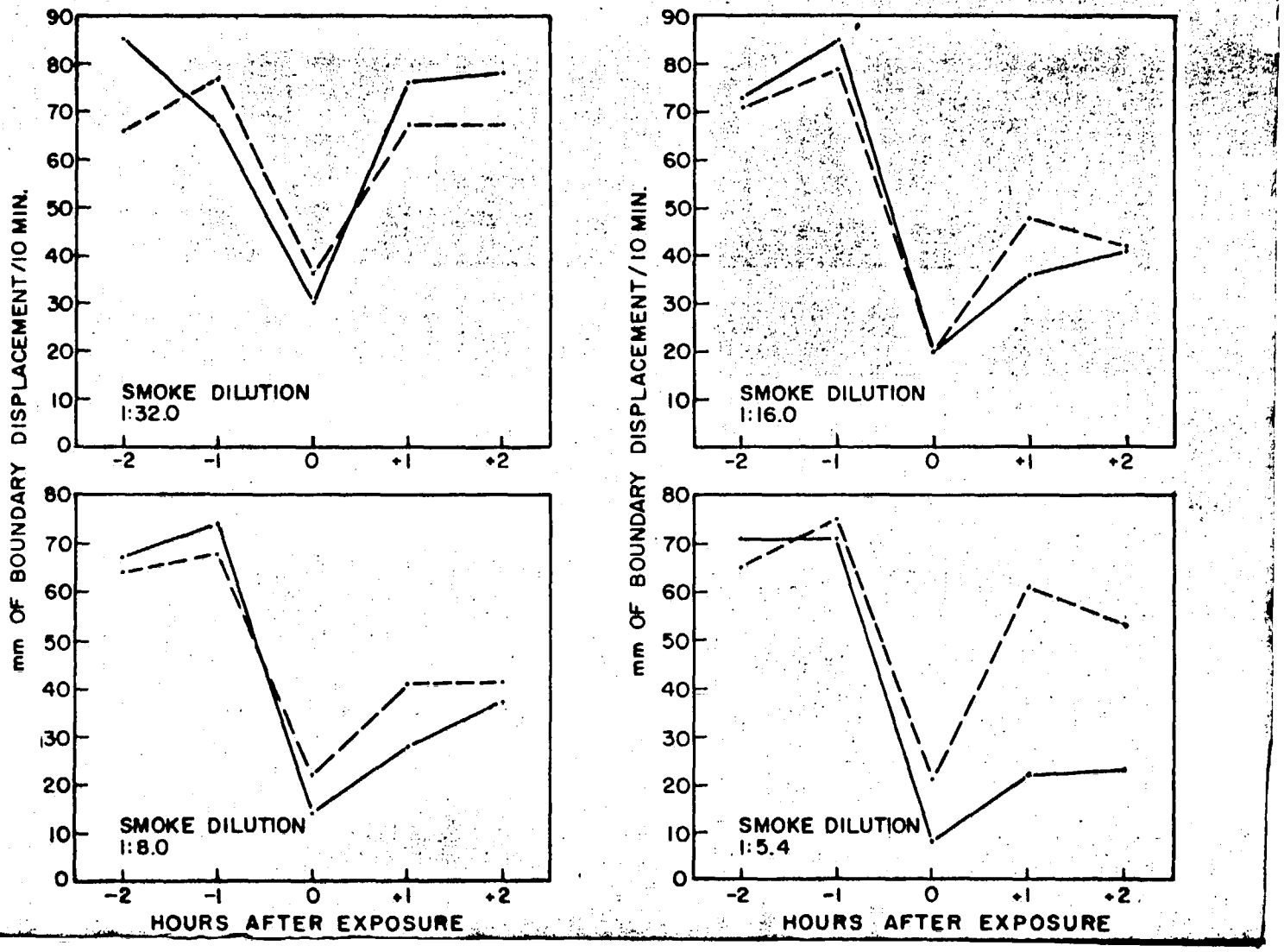
THE SALIVARY PERFORMANCE OF HIGH vs LOW SALIVATORS, UPON EXPOSURE TO FOUR WHOLE SMOKE DILUTIONS OF BRAND A



THE SALIVARY PERFORMANCE OF HIGH vs LOW SALIVATORS, UPON EXPOSURE TO FOUR WHOLE SMOKE DILUTIONS OF BRAND B



COMPARISON OF THE SALIVARY PERFORMANCE OF HIGH-SALIVATING C57BL/6J MICE UPON EXPOSURE TO FOUR DILUTIONS FROM BRANDS A —— vs B - - -



THE CALCULATED F-VALUES FOR "BRAND" AND "DILUTION" OBTAINED WITH "HIGH" AND "LOW" SALIVATORS AT THREE DIFFERENT TIMES AFTER EXPOSURE TO A WIDE OR NARROW DILUTION RANGE OF WHOLE SMOKES

Parameter	Time after Exposure (hours)	High Salivators		Low Salivators	
		6 Dil.	4 Dil.	6 Dil.	4 Dil.
"Brand"	0	1.51	1.21	0.01	0.00
	1	3.64	5.63*	1.11	0.79
	2	0.79	1.76	0.00	0.00
"Dilution"	0	22.82**	37.44**	8.95**	13.99**
	1	8.26**	9.71**	3.51**	5.33**
	2	5.39**	4.25**	2.19	2.36

* Significant at the 5% level of probability

** " " " 1% " " "